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Mouse Cytomegalovirus M33 Is Necessary and Sufficient in Virus-Induced Vascular Smooth Muscle Cell Migration

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Mouse cytomegalovirus (MCMV) encodes two potential seven-transmembrane-spanning proteins with homologies to cellular chemokine receptors, M33 and M78. While these virus-encoded chemokine receptors are necessary for the in vivo pathogenesis of MCMV, the function of these proteins is unknown. Since vascular smooth muscle cell (SMC) migration is of critical importance for the development of atherosclerosis and other vascular diseases, the ability of M33 to promote SMC motility was assessed. Similar to human CMV, MCMV induced the migration of mouse aortic SMCs but not mouse fibroblasts. To demonstrate whether M33 was required for MCMV-induced SMC migration, we employed interfering-RNA technology to specifically knock down M33 expression in the context of viral infection. The knockdown of M33 resulted in the specific reduction of M33 protein expression and ablation of MCMV-mediated SMC migration but failed to reduce viral growth in cultured cells. Adenovirus vector expression of M33 was sufficient to promote SMC migration, which was enhanced in the presence of recombinant mouse RANTES (mRANTES). In addition, M33 promoted the activation of Rac1 and extracellular signal-related kinase 1/2 upon stimulation with mRANTES. These findings demonstrate that mRANTES is a ligand for this chemokine receptor and that the activation of M33 occurs in a ligand-dependent manner. Thus, M33 is a functional homologue of US28 that is required for MCMV-induced vascular SMC migration.

Human cytomegalovirus (HCMV) is a ubiquitous betaherpesvirus that establishes a lifelong latent/persistent infection after primary infection. Although antiviral therapy has significantly reduced HCMV-related disease in individuals suffering from AIDS, HCMV infection remains a significant problem in congenital disease and transplant patients (27). HCMV infection has been associated with a number of vascular diseases, including atherosclerosis, restenosis following angioplasty, chronic rejection associated with solid organ transplantation, and, more recently, malignancies (7). However, the mechanisms involved in CMV-associated development of vascular disease are unknown (20, 21, 29).

The most-convincing evidence demonstrating that herpesvirus infections exacerbate vascular disease is exemplified in animal models. Marek's disease virus (MDV), a herpesvirus that infects fowl, was the first etiologic agent found to induce atherosclerosis (9, 10). MDV-infected chickens develop atherosclerotic lesions with histological features comparable to those of human vascular disease, which includes the finding of MDV antigens early in vascular lesions and late in smooth muscle cells (SMCs) at the periphery of the plaque. The advent of mouse models of atherosclerosis has dramatically improved the ability to study the effects of CMV infection on vascular lesion development. While wild-type (WT) mice appear to be resistant to the development of atherosclerosis, $ApoE^{-/-}$ mice are prone to develop the disease when fed a high-fat diet (25). Murine CMV (MCMV) infection of $ApoE^{-/-}$ mice accelerates the development of atherosclerosis by increasing the frequency of lesion formation and the severity of the atherosclerotic plaques (5, 14, 34). The crossing of $ApoE^{-/-}$ mice with other genetically altered mice has been employed to study the effects of host proteins in lesion formation. For example, MCP-1 and the receptor for this chemokine, CCR2, are important regulators of the monocyte infiltration involved in the formation of atherosclerotic plaques (3, 12). In a rat heart transplantation model, rat CMV (RCMV)-induced acceleration of chronic rejection is associated with increased infiltration of immune cells and enhanced chemokine expression (31). These and other similar findings suggest an important role for CMVs, chemokines, and chemokine receptors in the development of vascular disease.

All betaherpesviruses encode proteins with homologies to chemokines and/or chemokine receptors. For example, HCMV encodes four putative chemokine receptors: UL33, US27, US28, and UL78, with US28 being the most characterized (6). US28 is necessary and sufficient to induce the ligand-dependent migration of vascular SMCs (32), which involves the activation of the small G protein RhoA (22) and the protein tyrosine kinases focal adhesion kinase and Src (33). US28 was the first viral G protein-coupled receptor (GPCR) shown to mediate cellular motility, which is cell-type specific and provides a molecular basis for the correlative evidence that links

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HCMV to the acceleration of vascular disease. RCMV and MCMV encode two putative chemokine receptor homologues, R33 and R78 and M33 and M78, respectively. Disruption of either M33 or M78 results in reduced viral titers in salivary glands (8, 26). These findings and the conservation of virus-encoded chemokine receptors among betaherpesviruses suggest an important role for these molecules in the biology of these viruses.

Since vascular SMC migration is crucial for the development of atherosclerosis, and an animal model is unavailable for the study of HCMV, the ability of MCMV to induce the migration of vascular SMCs was determined. In this report, we demonstrate that although MCMV encodes two chemokine receptors (28), the putative CC chemokine receptor M33 is both necessary and sufficient in MCMV-induced SMC migration. While M33 has been shown to constitutively signal through phospholipase C-β and NF-κB (36), we report that recombinant mouse RANTES (mRANTES) enhanced M33-induced SMC migration and triggered the activation of the small G protein Rac1, as well as extracellular signal-related kinase 1/2 (ERK-1/2), demonstrating for the first time that mRANTES is a ligand of M33. Therefore, both MCMV and HCMV encode chemokine receptors that share similar abilities to induce the ligand-dependent migration of SMCs.

MATERIALS AND METHODS

Cells and viruses. NIH 3T3 fibroblasts and primary rat aortic SMCs (AoSMCs) were isolated from rat aortas (22). Due to the short life span in tissue culture of SMCs isolated from WT BALB/c mice, we isolated mouse AoSMCs from aortas of $p53^{-/-}$ mice that were subsequently cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and penicillin–streptomycin–L-glutamine (22). NIH 3T3 cells were used to prepare and determine the titers of stocks of the Smith strain of MCMV (ATCC) and MCMV-M33FlagGFP. MCMV-M33FlagGFP was constructed using the λ-derived linear recombination system in combination with the pSM3fr MCMV bacterial artificial chromosome in the *Escherichia coli* strain DY380 (provided by U. Koszinowski) (35). MCMV-M33FlagGFP contains a green fluorescent protein (GFP) expression cassette under the control of the EF1-α promoter, which was cloned into the IE2 promoter region, and a C-terminal Flag epitope (Flag) was cloned in frame with M33. MCMV-ΔM33 (MCMV with M33 deleted) was constructed using the λ-derived linear recombination system by placing multiple stop codons in frame with the M33 start codon. MCMV-M33Rev (MCMV with revertant M33) was constructed by replacing the ΔM33 stop codons with WT M33 sequences by linear recombination. The recombinant viruses have been checked for insertion by restriction analysis, Southern blotting, and sequencing. All of the recombinant viruses grew to levels similar to that of WT MCMV Smith in mouse fibroblasts. NIH 3T3 cells were used to prepare and determine the titers of MCMV stocks.

M33 adenovirus construction. The adenovirus (Ad) vector expressing M33 containing a C-terminal Flag epitope tag was constructed as previously described (13, 32). Briefly, M33 containing a C-terminal Flag epitope tag was constructed by subcloning the cDNA fragment into pAdTet7 (13), which contains the *tet*-responsive enhancer within a minimal CMV promoter followed by the simian virus 40 late poly(A) cassette. Recombinant adenoviruses were produced by cotransfection of pAdTet7-M33C-Flag and Ad5-ψ5 DNA into 293-Cre cells (13). Recombinant adenoviruses were expanded on 293-Cre cells, and their titers were determined by limiting dilution. Expression of M33 was driven by coinfection with Ad-*trans* expressing the "Tet-off" transactivator as previously described (32).

siRNA. The M33-specific silencing inhibitory RNAs (siRNAs) (M33-345-365, AACCGCAUCUAUCGCAGCUCG and M33-387-409, AACCUGUACUUUG CGAACCUG) and lamin A/C-specific siRNA (AACUGGACUUCCAGAAGA ACA) were obtained ready to use from Dharmacon (Lafayette, CO) as option A4 (5′-end deprotected, annealed, and desalted). Selected sequences were searched using BLAST to ensure that the siRNAs were specific for their target sequences.

The methods of siRNA transfection were the same whether used in assays for monitoring virus replication, protein expression, or SMC migration. Briefly, mouse AoSMCs were cultured on 12-well dishes (or T75 flasks for SMC migration assays). Prior to transfection, the cells were washed twice with Opti-MEM. Various concentrations (1 to 50 nm) of siRNA M33-345, M33-387, or lamin A/C along with 2 μl of oligofectamine (GibcoBRL) were diluted in Opti-MEM (200 μl). The solution was incubated for 20 min and then added to the cells for 4 h, at which time they were supplemented with 10% fetal calf serum-Dulbecco's modified Eagle's medium. Sixteen hours posttransfection, cells were infected with MCMV-M33FlagGFP at a multiplicity of infection (MOI) of 1. To assess the effects of siRNA on MCMV entry, MCMV-M33FlagGFP-infected, GFP-positive cells were visualized using a Nikon TE300 microscope (magnification, ×20) and enumerated by counting multiple fields. Multistep growth curves were examined to determine the effects of siRNA on virus replication. Culture supernatants were harvested daily, and limiting dilution plaque assays were used to quantitate infectious viruses in each sample. For protein expression experiments, the cells were lysed 24 h postinfection (hpi) in 2× Laemmli's sample buffer and analyzed by Western blotting as described below. For SMC migration experiments, siRNA-transfected SMCs were transferred to transwells and infected with MCMV at an MOI of 1. Migration assays were then performed as described below.

SMC migration assays. Cell migration assays were performed as previously described (22, 32, 33). Briefly, 1×10^5 cells were added to the upper well of a transwell (3.0-μm pore size; Costar Corning, Cambridge, MA). Cells were serum starved and infected with MCMV (MOI = 1). Infection inserts were washed and transferred to new 12-well tissue culture plates. Cells migrating to the lower chamber were counted at 48 to 72 hpi by using a Nikon TE300 microscope at a magnification of ×10. Experiments were done in at least triplicate wells, and 10 random fields were read from each well.

Immunofluorescent microscopy. For immunofluorescent analysis, samples were washed with phosphate-buffered saline (PBS) and fixed with 2% foscarnet in PBS. For intracellular staining, the samples were permeabilized and blocked in intracellular staining buffer (ISB) (1 g bovine serum albumin, sodium azide, 0.5% Triton X-100, and 500 ml PBS) with 10% normal goat serum for 20 min. The primary antibody (diluted in ISB) was incubated for 2 h. Anti-Flag antibodies (M2; Sigma) were used to visualize M33Flag-tagged proteins. Samples were washed with ISB and incubated with fluorescein isothiocyanate or L-rhodamine-conjugated secondary anti-mouse or anti-rabbit antibodies (BioSource International, Camarillo, CA), diluted in ISB for 1 h. Samples were washed with ISB, mounted, and visualized using a Nikon TE300 microscope. Mouse AoSMCs were stained with either anti-α-SMC actin (1:250) or M2 anti-Flag monoclonal antibody (1:500) diluted according to the manufacturer's instructions. SMCs were then incubated with secondary fluorescence-conjugated antibodies, and photomicrographs were obtained at ×60 magnification (Nikon; TE300).

Western blot analysis. Western blotting of cellular lysates for viral and cellular proteins was accomplished as previously described (22, 33). Briefly, mock-infected or MCMV-M33FlagGFP-infected NIH 3T3 cells (5×10^5) were lysed in 2× Laemmli's sample buffer (17). Samples were analyzed by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and proteins were transferred to Immobilon-P membranes (Millipore). The blots were blocked with 3% milk in Tris-buffered saline–T buffer (100 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1 h. The M2 anti-Flag antibody (diluted to 1:2,000 in Tris-buffered saline–Tween 20) was used for primary detection of M33-Flag proteins with a secondary anti-mouse antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). Anti-MCMV antibodies recognizing the MCMV immediate early protein 1 (IE1) or the early protein pp50 (M44) were used to determine the extent of viral protein expression. Chemiluminescence and autoradiography were used for final protein detection on Kodak MR or Biomax light film.

Rac activation assay. The CDC42/Rac-interactive binding domain (CRIB) of Pak1 (amino acids 67 to 150) was expressed as a glutathione *S*-transferase (GST) fusion (GST-CRIB; J. Scott, OHSU) in the *E. coli* strain BL21 as previously described (16, 19). Upon our obtaining an optical density of 0.6 to 0.8, bacteria were induced overnight with IPTG (isopropyl-β-D-thiogalactopyranoside; 1 mM), and clarified supernatants were bound to glutathione-linked 4B-CL Sepharose (Amersham). The beads were washed in PBS, followed by three washes in Rac wash buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 150 mM NaCl, and 10 mM $MgCl_2$), with a final equilibration wash using Rac1 lysis buffer (50 mM Tris, pH 7.2, 1% Triton X-100, 500 mM NaCl, 10 mM $MgCl_2$, 0.5% sodium deoxycholate, and 0.1% SDS). For Rac activation assays, rat AoSMCs were cultured on 15-cm dishes and upon achieving 75% confluence were serum starved for 18 h prior to being infected with Ad-M33CFlag and/or Ad-*trans* at an MOI of 1,000. The cells were stimulated with mRANTES (10 ng/ml) at 16 h postinfection and harvested in Rac lysis buffer at 0 (unstimulated), 5, 10, or 30 min. Prior to

administration of GST-CRIB beads, a sample of each cell lysate was analyzed for total input Rac1 and for ERK-1/2 activation by Western blotting for Rac1, phospho-ERK-1/2 (Thr202/Tyr204), and total ERK-1/2 (Cell Signaling Technologies). Cell lysates were incubated with GST-CRIB beads (80 μl of a 1:1 slurry) for 45 min at 4°C and then washed four times with 1 ml of Rac1 wash buffer. The final pellet was resuspended in 60 μl of 2× Laemmli's sample buffer, subjected to 12% SDS-polyacrylamide gel electrophoresis, transferred to Immobilon-P membranes, and probed for Rac1.

RESULTS

MCMV-M33 mediates mouse SMC migration. We have previously demonstrated that the HCMV-encoded chemokine receptor US28 is capable of inducing SMC migration, thus providing a molecular link between HCMV and the acceleration of vascular disease (32). Since SMC migration is a fundamental process in the development of vascular diseases, we determined whether MCMV infection of SMCs resulted in cellular migration similar to SMC motility induced by HCMV (32). To accomplish this, AoSMCs were isolated from $p53^{-/-}$ mice, and to confirm their phenotypes, cells were stained with antibodies directed against α-SMC actin (Fig. 1A). Although the isolated SMCs stained α-SMC actin, staining for the endothelial cell marker von Willebrand's factor yielded negative results, indicating that the SMC population was not contaminated with endothelial cells. Subsequently, AoSMCs were cultured in transwell dishes for migration assays, and infection with MCMV induced their migration (Fig. 1D). MCMV encodes two chemokine receptors, M33 and M78, and M33 is similar to the HCMV-encoded chemokine receptor US28 (28). Similar to US28 and U12 (M33 positional homologue in human herpesvirus 6 [HHV-6] and HHV-7), M33 is predicted to bind CC chemokines, including RANTES. Thus, an adenovirus vector expressing a C-terminal Flag-tagged version of M33 (Ad-M33Flag) was generated to determine whether M33 was responsible for MCMV-induced AoSMC migration. When expressed in mouse SMCs, M33 localized to intracellular vesicles, similar to US28 (Fig. 1B) (32, 33). Ad-M33CFlag expression in mouse SMCs resulted in cellular migration comparable to that of MCMV-infected cells (Fig. 1C). These results indicate that M33 is sufficient for SMC migration and that this chemokine receptor is a functional homologue of US28 (Fig. 1D). The induction of SMC migration by M33 was independent of an exogenous source of chemokine ligands, suggesting that this chemokine receptor is either constitutively active or that SMCs produce ligands for M33. Previous experimental evidence supports the latter hypothesis, as in vitro-cultured human SMCs constitutively produce the US28 ligand MCP-1, which is capable of promoting migration in the absence of exogenously added chemokines. The addition of neutralizing MCP-1 antibodies abrogates US28-mediated SMC migration, and the migration phenotype can be rescued upon the addition of RANTES (32). Similarly, a consistent dose-dependent increase in the migration of M33-expressing mouse AoSMCs was observed with the addition of recombinant mRANTES to the lower chamber, indicating that M33 responds to ligand stimulation to promote cellular motility.

As an alternative approach to demonstrating that M33 is the only MCMV gene involved in promoting SMC migration, we used siRNA technology to block expression of M33 in the context of full virus replication. Two different siRNA molecules were designed against MCMV-M33 sequences and designated M33-345 and M33-387 based on their relative positions in the M33 gene. In order to optimize siRNA transfection procedures, we also obtained a fluorescent (fluorescein isothiocyanate)-tagged control oligonucleotide to the cellular protein lamin A/C, which was transfected into >90% of the cells (data not shown).

Use of inhibitors, including siRNA, can have deleterious effects on viral gene expression and replication. To test whether siRNA transfection negatively impacted MCMV infection, cells were transfected with either the lamin A/C control siRNA or one of the two siRNAs directed against M33 and then infected with MCMV-GFP at an MOI of 1. As assessed by fluorescence, the siRNA molecules did not affect the ability of MCMV to infect mouse fibroblasts (data not shown). These findings were confirmed by determining MCMV replication in the presence of each of the siRNAs by using multistep growth analysis (Fig. 2). MCMV replicated to levels in mouse fibroblasts treated with any of the siRNA reagents equivalent to those of mock-transfected cells, thus providing a means of specifically knocking down genes during viral infection, without affecting in vitro MCMV replication.

Currently, antibodies directed against M33 are not available. Thus, in order to monitor M33 protein expression during MCMV infection, a recombinant version of the Smith strain of MCMV containing a Flag-tagged version of M33 and expressing GFP under the control of the constitutive EF1α promoter was constructed (MCMV-M33FlagGFP). MCMV-M33FlagGFP displays replication characteristics that parallel those of MCMV-GFP (data not shown). To determine the kinetics of M33 protein expression, NIH 3T3 cells were infected with MCMV-M33FlagGFP at an MOI of 1. Cells were harvested in sample buffer at the times indicated in Fig. 3A, and expression of IE1, the early protein M44 (pp50), and M33 (Flag) was assessed by Western blot analysis (Fig. 3). As expected, robust IE1 expression commenced by 4 hpi and was sustained for the duration of the experiment. The DNA processivity factors M44 and M33 were expressed with similar early kinetics (detectable by 12 hpi).

The efficacy of siRNA as a means of reducing protein expression varies depending on the target gene, cell type, and individual siRNA. To determine whether siRNAs directed against M33 blocked protein expression, mouse fibroblasts were transfected with various concentrations (0, 5, 10, or 25 nM) of the three siRNAs. Cells were then infected with MCMV-M33FlagGFP for 24 h. As shown in Fig. 4A and B, Western blotting for M33 Flag protein expression was quantitated and normalized to the protein levels of M44 (pp50, MCMV early protein). In accordance with our findings that MCMV replication was not affected by siRNA transfection, the levels of pp50 in the siRNA-treated samples were unchanged compared to those of mock-transfected and MCMV-infected cells. Interestingly, the two oligonucleotides specific for M33 differed in their abilities to block M33 protein expression. The M33-387 oligonucleotide was more effective in blocking M33 protein expression than was the M33-345 oligonucleotide, reducing M33 expression levels to 40% versus 100% of the lamin control (Fig. 4B). To confirm our findings that M33 was responsible for SMC migration induced by MCMV, siRNA molecules were tested in SMC migration assays.

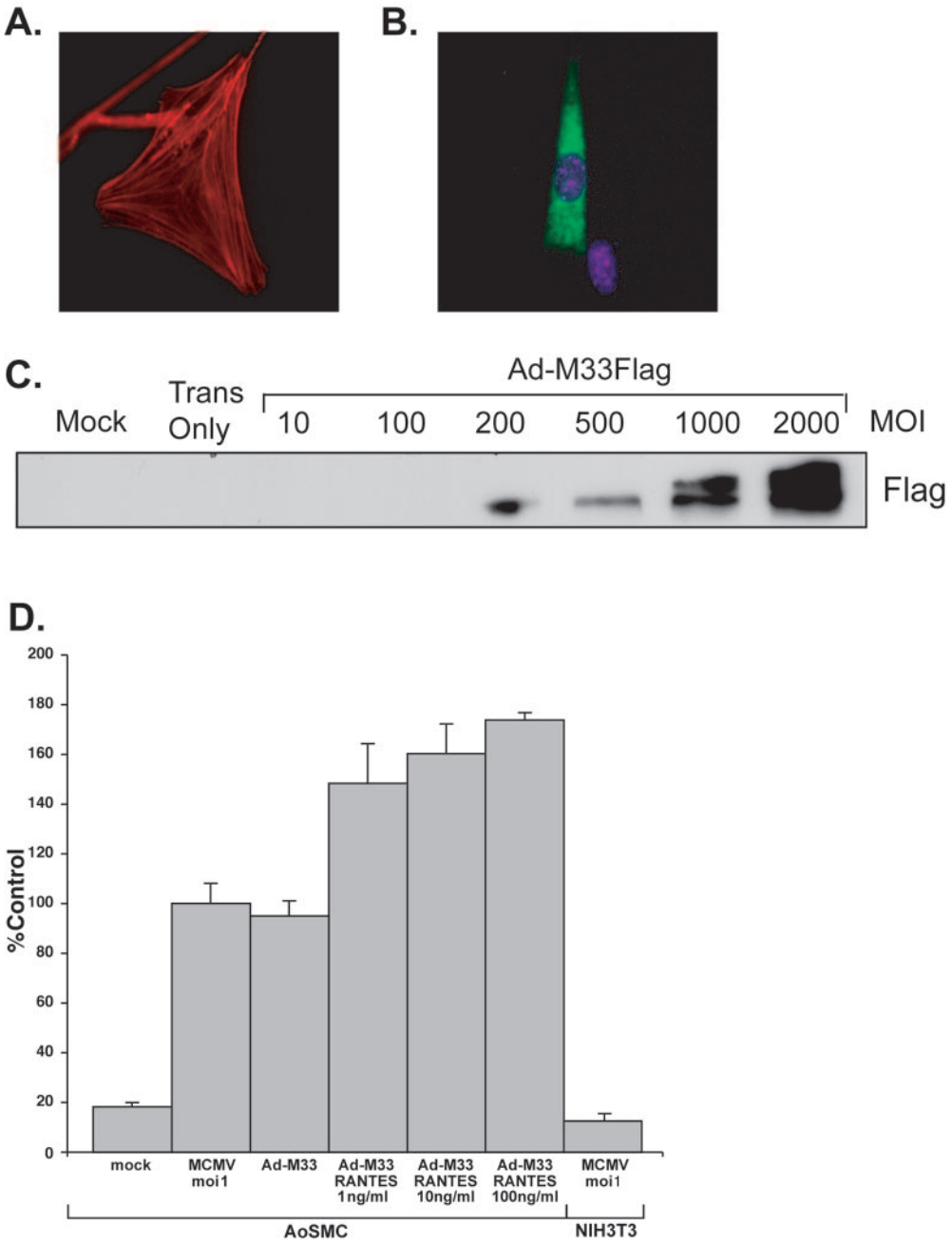

FIG. 1. MCMV-M33 induces vascular smooth muscle cell migration. (A) Mouse vascular smooth muscle cells isolated from the aorta of $p53^{-/-}$ mice were stained for the SMC marker α-SMC actin (red).(B) To examine M33 protein expression, mouse AoSMCs were coinfected with an adenovirus vector expressing M33Flag (Ad-M33Flag) and the "Tet-off" tetracycline transactivator (Ad-*trans*). At 24 hpi, cells were fixed and then stained using an antibody directed against the Flag epitope (green). Nuclei were stained with the Hoescht DNA stain (blue). (C) AoSMCs were infected with various concentrations of Ad-M33Flag in order to determine the optimal levels of protein expression. Cell lysates from Ad-M33Flag-infected cells were harvested at 24 hpi and probed for M33 by Western blotting by using an antibody directed against the Flag epitope. (D) For the SMC migration assay, mouse aortic SMCs or NIH 3T3 cells (10^5 cells per well) were plated onto transwells and infected with either MCMV or Ad-M33Flag. Ad-M33Flag-infected cells were treated with increasing concentrations of RANTES in the lower well. Migrating cells were enumerated by microscopy 48 to 72 hpi.

AoSMCs were transfected in triplicate with either control lamin A, M33-345, or M33-387 siRNAs as described above. Cells were then infected with MCMV-M33FlagGFP, and 16 hpi, the cells were divided and transferred into 12-well transwell dishes. Treatment with the M33-387, but not the M33-345 or lamin control, siRNA abrogated MCMV-M33-mediated SMC migration (Fig. 4C). The effects of M33-387 on MCMV-M33-mediated SMC migration were consistent with our findings that this oligonucleotide effectively blocks M33 protein expression. To confirm the requirement of M33 in MCMV-

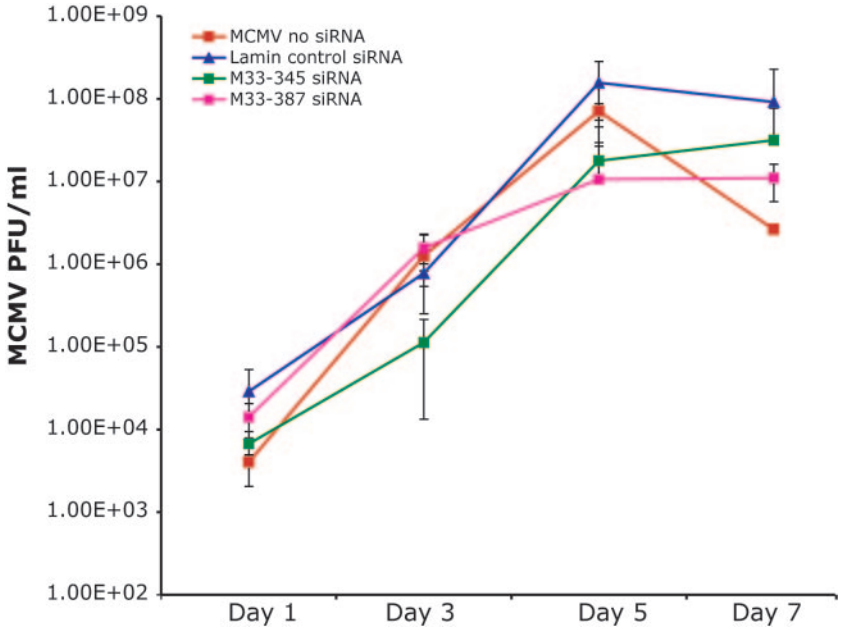

FIG. 2. siRNA transfection does not interfere with MCMV replication. MCMV multistep growth curves were performed on mouse fibroblasts transfected with M33-345, M33-387, or lamin siRNA and then infected with MCMV-GFP at 24 h posttransfection. Cell supernatants were analyzed by plaque assays for the presence of infectious MCMV.

induced SMC migration, we performed SMC migration assays in $p53^{-/-}$ SMCs that were either mock infected, infected with MCMV deleted for M33 (MCMV-ΔM33), or infected with the recombinant M33 revertant strain of MCMV (MCMV-M33Rev) (Fig. 4D). MCMV-ΔM33-infected SMCs displayed reduced cellular migration compared to MCMV-M33Rev-infected SMCs. In addition, the levels of reduction, while not complete, are consistent with our siRNA M33 depletion experiments described above. Therefore, we conclude that M33 mediates SMC migration and is a functional homologue of US28 in MCMV.

M33 activation of Rac1 and ERK-1/2. Although we have demonstrated a function for M33, the signaling pathways activated by M33 and the ligands that bind this viral GPCR are unknown. We have recently demonstrated that US28 signaling through RhoA is critical for the ability of US28 to induce SMC migration (22). Rac1, an additional member of the Rho-like G protein family, is a key mediator of cellular migration (11). To assess the ability of M33 to activate Rac1, active Rac pull-down assays were performed using glutathione-linked Sepharose beads bound with GST-CRIB. This fusion protein binds only to Rac1 in the active GTP-bound state (16, 19). Serum-starved rat AoSMCs expressing M33 and/or the *tet* transactivator were stimulated with 10 ng/ml of recombinant mRANTES for 0 (unstimulated), 5, 10, or 30 min. GST-CRIB was administered to precleared cell lysates, and active Rac1 associated with GST-CRIB was determined by Western blotting for Rac1. To ensure that equivalent amounts of cellular proteins were used in each assay, precleared lysates were analyzed by Western blotting for the presence of Rac1 prior to the addition of GST-CRIB. SMCs expressing M33 and treated with recombinant mRANTES demonstrated a kinetic activation of Rac1, with peak activation occurring 10 min after ligand stimulation (Fig. 5). Similarly, the addition of RANTES to M33-expressing

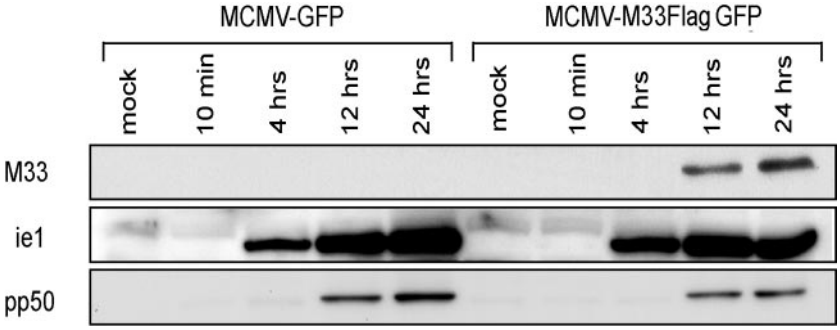

FIG. 3. M33 protein is expressed with early kinetics during MCMV infection. Mouse fibroblasts were infected with MCMV or MCMV-M33FlagGFP at an MOI of 1. To determine the timing of M33 expression, cell lysates were probed by Western blotting for M33Flag, IE1, or the early protein pp50 (M44) at the times indicated above the blot.

cells, but not control AoSMCs, induced the phosphorylation/activation of ERK-1/2, as determined by Western blotting for ERK-1/2 using phosphospecific antibodies. Therefore, similar to US28, M33 activates ERK-1/2, as well as small G proteins that are known to be important in cellular migration in a ligand-dependent manner.

DISCUSSION

In this report, we demonstrate that similar to our findings with HCMV and the HCMV-encoded chemokine receptor US28, MCMV infection of vascular SMCs induces their migration (32). We used siRNA to knock down M33 expression, which had no effect on viral growth kinetics, representing a viable technique for specific gene knockdown in the presence of viral replication. While M33-siRNA treatment blocked AoSMC migration in MCMV-infected cells, expression of M33 was sufficient to induce migration to levels similar to MCMV infection. The addition of RANTES to M33-expressing SMCs enhanced migration, resulting in the activation of Rac1. To our knowledge, this is the first demonstration of a ligand for M33. Furthermore, these findings also demonstrate that this viral GPCR signals in a ligand-dependent manner. Together, these findings suggest that M33 is a functional homologue of US28 and that M33 is required for MCMV-induced SMC migration.

SMC migration from the media into the neointimal space is a hallmark of vascular lesion formation, suggesting that pathogen-mediated acceleration of vascular disease involves enhanced accumulation of SMCs in the lesion. A reduction in apoptosis caused by HCMV infection of SMCs could lead to an accumulation of these cells at sites of vascular injury. CMV infection of HeLa cells inhibits tumor necrosis factor alpha-induced apoptosis (37). An explanation for this block in apoptosis is the finding that the HCMV IE1 binds and inactivates the tumor suppressor gene p53 (29). Another mechanism of cellular accumulation occurs through SMC proliferation at the site of vascular injury. CMV infection of endothelial cells induces the release of growth factors and cytokines, including fibroblast growth factor and platelet-derived growth factor BB, which are potent stimuli of SMC proliferation (30). Furthermore, HCMV infection up-regulates expression of the CC chemokine RANTES in SMCs and fibroblasts (23, 32). We have previously demonstrated that infection of human SMCs with HCMV induces migration, which is dependent upon expression of the virus-encoded chemokine receptor US28, and binding of the CC chemokines RANTES or MCP-1 (32). Similarly, in the current report, we have shown that MCMV-M33 induces mouse SMC migration. We hypothesize that HCMV infection enhances SMC migration preferentially towards sites of vascular injury due to expression of virus-encoded chemokine receptors. The resultant SMC accumulation in the vessel intima leads to neointimal hyperplasia and vessel narrowing. siRNA-mediated ablation of M33 protein expression during MCMV infection confirmed the requirement of this chemokine receptor in MCMV-induced SMC migration.

β-Chemokine receptors in betaherpesviruses. While others have demonstrated that M33 can signal independently of exogenous ligands (36), we demonstrate that RANTES binding to M33 enhances SMC migration and activates Rac1 and ERK-1/2. These findings indicate that RANTES is a potent ligand for M33. The ligands for HCMV UL33, the M33 positional homologue, are unknown. Deletion of US27 and US28 is required to prevent RANTES binding in HCMV-infected fibroblasts, suggesting that UL33 does not bind RANTES (2). Interestingly, the presence of a β-chemokine receptor is common for all betaherpesviruses, as HHV-6 and HHV-7 also encode β-chemokine receptors (15, 24). Additionally, a recent finding indicates that the gene carrying HCMV also encodes a

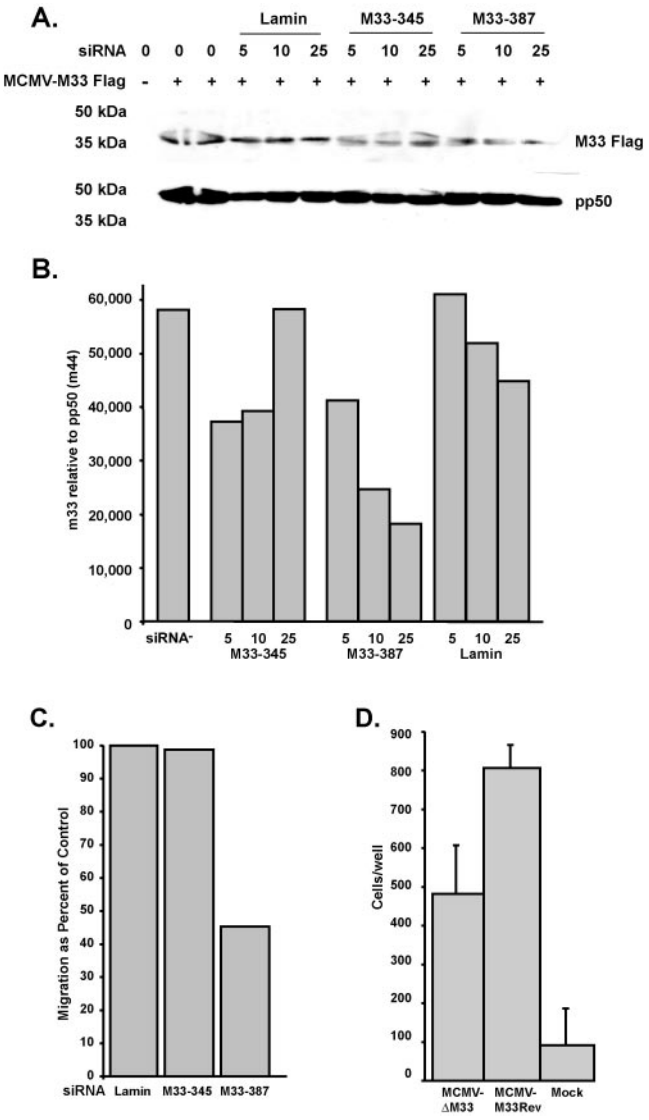

FIG. 4. M33-specific siRNA blocks protein production and vascular SMC migration. (A) Inhibition of M33 protein synthesis in MCMV-M33FlagGFP-infected mouse fibroblasts transfected with 5, 10, or 25 ng siRNA (lamina, M33-345, or M33-387). M33 protein (*y* axis) was measured relative to pp50. (B) Quantitation of M33 protein inhibition. M33 protein was measured relative to the MCMV early protein pp50. (C) MCMV-infected AoSMCs were treated with siRNA (lamina, M33-345, or M33-387) and subjected to migration assays. Percent migration was determined by comparing M33 siRNA-treated cells to the lamin control. Migrating cells were enumerated by microscopy at 48 to 72 hpi. (D) For the SMC migration assay, mouse aortic SMCs (1×10^5 cells per well) were plated onto transwells and infected with either MCMV-ΔM33 or MCMV-M33Rev. Migrating cells were enumerated by microscopy 48 to 72 hpi.

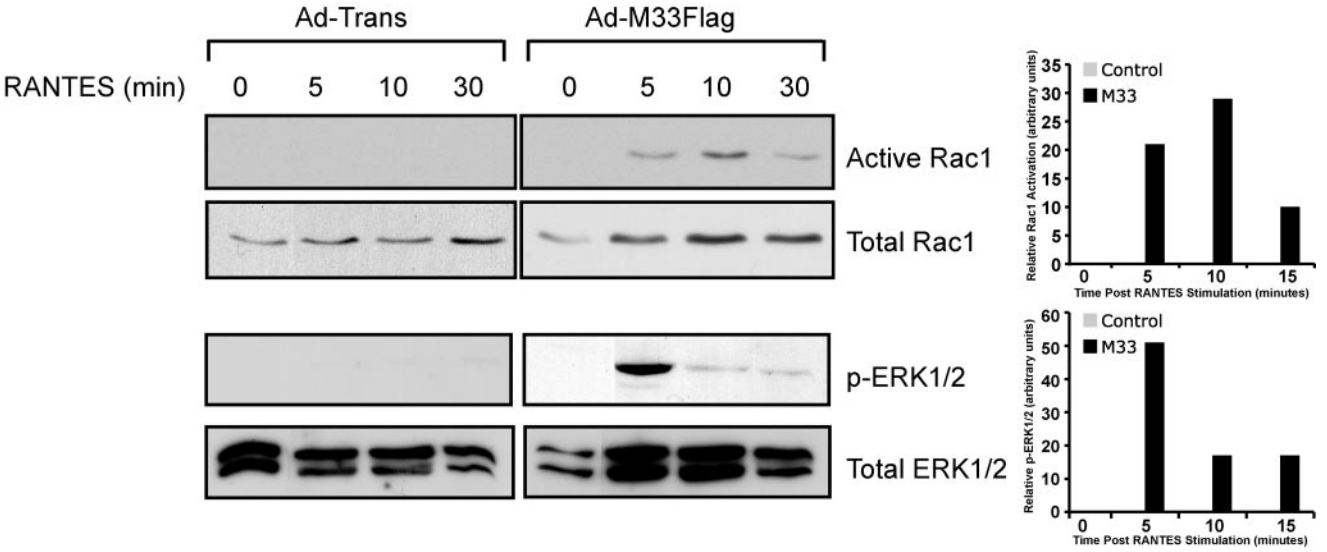

FIG. 5. RANTES stimulation of M33 activates Rac1. AoSMCs expressing M33 and/or *trans* were stimulated with 10 ng/ml recombinant mRANTES. Lysates were baited with GST-CRIB for active Rac1 pull-down assays. Active and total Rac1 were determined by immunoblotting using a Rac1-specific antibody. ERK-1/2 phosphorylation/activation was determined by Western blotting using phosphospecific antibodies. Total input protein was detected by Western blotting for Rac1 and ERK-1/2. The relative levels of active Rac1 and ERK-1/2 were normalized to the total input protein using NIH ImageJ. p-ERK, phospho-ERK.

soluble RANTES binding protein (4). The transcript for this gene is packaged in the virion, and the protein is expressed immediately following infection.

What is the function of M33 in MCMV pathogenesis? While we have demonstrated a role for M33 in the induction of vascular SMC migration, this finding does not preclude M33 from having other functions. Deletion of either MCMV-M33 or RCMV-R33 has deleterious effects on virus replication in salivary glands, suggesting that both are important for viral persistence in the host (1, 8). Similarly, deletion of mouse HV-68 viral GPCR does not affect acute-phase viral replication but prevents reactivation from latency (18). Whether viral chemokine receptors are utilized as sensors to monitor the host inflammatory response to ensure that the extracellular environment is amenable for replication is unclear. In support of this hypothesis, HCMV reactivates in times of immune stress when chemokine ligands are induced, promoting monocyte differentiation into HCMV infection competent macrophages.

SMC migration induced by CMV-encoded chemokine receptors has important implications for several inflammatory vascular diseases, including restenosis, transplant vascular sclerosis, and atherosclerosis, which involve endothelial cell damage and inflammatory cell infiltration, followed by SMC accumulation resulting in stenosis of the vessel. Although HCMV has been linked to these vascular diseases, the pathogenic features of these disease processes are complex and multifactorial. The accumulation of SMCs in the intima is hypothesized to involve both migratory and proliferative events. Our studies suggest a novel mechanism for the accumulation of SMCs in vascular lesions, whereby virus-encoded chemokine receptors might induce SMC migration to sites of atherogenesis. Further investigation of the signaling pathways involved in CMV-induced SMC migration is warranted in order to develop strategies to prevent and treat CMV-associated vascular diseases.

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